United States Department of the Interior

1 November 2010

Dear Dr. Ash,

Please find associated with this letter our revised manuscript, **A bacterium that can grow by using arsenic instead of phosphorus**, for potential publication in *Science*. We have addressed and incorporated your suggestions and comments and those of the three reviewers and our responses follow this cover letter. As we previously communicated, we have incorporated additional data resulting in a revised figure (Figure 2) to further support and strengthen our conclusions. These data were generated and verified in close collaboration with additional co-authors (G. Gordon, J. Pett-Ridge and P. Weber, see information below).

We have also uploaded a potential cover image. This is an NanoSIMS image of the bacterium we isolated, strain GFAJ-1 of the Halomonadaceae, grown under conditions of high arsenic with no added phosphorus. This heat map indicates intracellular regions of high ion $^{75}As^{-}$:$^{12}C^{-}$ ratio and serves to demonstrate one of the major findings presented in our paper in a striking visual manner (note, this image can be found in our manuscript as Fig. 2B). Also, we have uploaded a single pdf file containing all of our SOM information: 1197258s.pdf, 303 kB.

I will be traveling Nov. 3 - 7, 2010 but will have email and mobile phone access the entire time. Please do not hesitate to contact me if there are any questions regarding our manuscript.

We hope you find our responses and edits satisfactory for the dissemination of our findings by *Science* and appreciate the recognition by the editors of the potential importance of our research to the community.

Sincerely yours,

Felisa Wolfe-Simon

lead and communicating author

[redacted]

U.S. Geological Survey
345 Middlefield Rd MS480
Menlo Park, CA 94025 USA
office: [redacted]
mobile: [redacted]

Coauthors:

Jodi Switzer Blum [redacted]

Jennifer Pett-Ridge [redacted]

Paul C.W. Davies [redacted]

Thomas R. Kulp [redacted]

John F. Stolz [redacted]

Ariel D. Anbar [redacted]

Gwyneth W. Gordon [redacted]

Samuel M. Webb [redacted]

Ronald S. Oremland [redacted]

Shelley E. Hoeft [redacted]

Peter K. Weber [redacted]

[redacted]

[redacted]

Responses to comments by reviewers
(Editor/Reviewers' comments in **bold** and authors' responses in *italic*)

Editor comments:

1. A concluding sentence? (to abstract)

Added.

2. p6: Might the high salt conditions bring the activities of P and As together?

In a previous manuscript we evaluated the similarity in terms of speciation of P and As over a gradient of pH conditions (1). We used a model, in MINEQL, to estimate the behavior of these two species and they have remarkably similar pKa values. Thus, we do not feel that salt would affect one more than the other necessarily, and as with the speciation over a pH gradient, they should behave quite similarly.

3. p6: Is this organism sequenced? Does this substitution affect sequencing?

This organism is not sequenced and we intend to submit it to the community genome sequencing project and/or apply for funding for pyrosequencing it. That said, we have no idea if the substitution will affect sequencing. However, we were indeed able to amplify a 16s gene fragment and so my impression is that As substitution does not seem to affect the recognition of taq polymerase. Given our x-ray data the structure looks like it should be exceedingly similar and thus an enzyme would be able to recognize a site for amplification. As for primers, again if the structure is so similar or even identical and the bases are the same- then base pairing and polymerization should proceed as we would expect. And it did.

4. Do you think that organisms would have a degree of As substitution in the "wild" or does it exclusively prefer P it its available?

For this manuscript, we wanted to show the potential of this biochemistry as a proof of concept. That is, we pursued the least difficult isolate and growth condition. As we show, this microbe can and will grow well on P however we have additional data that is in preparation for a followup paper with cells growing +As/+P and they grow better. Understanding how they partition the As and P under that condition may help us understand a "constitutive" condition that may be relevant in the "wild". We would of course love the chance to look for the abundance of this microbe or others like it in the field to determine the answer to this question in situ.

5. p8: Evidence [that hydrocarbon would stabilize AsDNA]? Why?

Arsenic esters have much higher hydrolysis rates than phosphate esters (2-4). These authors (3) also show the effect of a non-aqueous solvent (e.g. ethanol and others) that leads to a slower hydrolysis rate. Furthermore, an idea has been championed by Steve Benner and others and put forth by Schoffstall (5-7) that a non-aqueous solvent for life would actually be an easier "origins" serum. In particular, they favor formamide but there has been speculation regarding the hydrocarbons present on other celestial bodies like Titan, a moon of Saturn (8). Thus if AsDNA is more easily hydrolyzed cells may have a number of mechanisms to stabilize it including the potential for a region of the cell that is less aqueous (i.e. hydrocarbon rich) and/or proteinaceous structures for additional stability and protection.
In addition, the most relevant experiments to date have only specifically looked at the exchange or hydrolysis of the following (3):

$OAs(OR)_3 + H_2O \rightarrow OAs(OH)(OR)_2 + ROH$ (1)

$OAs(OH)(OR)_2 + H_2O \rightarrow OAs(OH)_2(OR) + ROH$ (2)

Where R = methyl, ethyl, n-pentyl and isopropyl. There are very few studies done examining the fine detailed kinetics of arsenate ester hydrolysis where arsenate is bound to other biologically relevant compounds (For an interesting approach that is worth reproducing see 9).

Review 1

The manuscript by Wolfe-Simon et al. demonstrates for the first time that a microorganism is able to use arsenic in place of phosphorous to sustain growth and life. This was done by using a rather simple initial selection on synthetic growth medium followed by a more in depth analysis of the isolated organism with regard to the path of arsenate from uptake to incorporation into various cellular fractions using ICP-MS, 73As labeling and X-ray absorption near edge spectroscopy (XANES).

The results are exceptional as they show that arsenate, yet believed to be highly toxic for most organisms, in GFAJ-1, a member of the Halomonadaceae, can substitute for the lack of phosphate, a major building block for various macromolecules present in all cells, namely nucleic acids, lipids and proteins.

The methods applied are straight forward. The most surprising and acknowledgeable aspect of the work is its simple approach.

I have only a few minor points regarding the overall presentation:
On page 3 it is said: "..., usually metal(loid)s present in trace quantities that serve critical cellular functions, such as enzyme co-factors."
Some metalloids are indeed present in rather high amounts, not only as trace elements. And Si and B perform structural roles rather than function as co-factors for enzymes.

Metalloids appear to have intermediate functions between non metals and metals, just as their position in the periodic table suggests. (Even an alternative organic chemistry based on Si instead of C was suggested. But this is yet pure theory.)

While we agree with the reviewer, we wanted to point out the most salient and major constituents of life, CHNOPS. Other "trace" nutrients have not been shown to be absolutely required for all life by weight percent of the organism (Si for example is only required in a selected array of organisms like diatoms and grasses) as compared to these six elements. Indeed, Si-based life would be spectacular to discover.

Further on page 3 it says: "The most common form of P in biomolecules is phosphate." This is confusing. Phosphorous occurs as free phosphate, phosphatidyl or phosphoester. What all (not most of) these forms and residues have in common is the oxidation state of P. If the authors are aware of any exception, then please make a reference!

The reviewer makes an excellent point, P is in the +5 oxidation state in biology. In all these molecules, P is bound to 4 oxygen atoms (10) and so we feel that indeed it is correct to say that "phosphate" is the most common- but perhaps it is more accurate to say "...in biology" or "...in life". We changed this to read "...in biology". There are other molecules like phosphonates but these organic phosphorus containing compounds are not nearly as abundant in biological systems on a big picture scale.

"..., because metabolic pathways intended for PO43- cannot distinguish between the two (PO43- and AsO43-) molecules". This is only part of the story: Many organisms possess arsenate reductases that can very well discriminate arsenate from phosphate, thereby diverting arsenate from phosphate metabolism. It would be interesting to know, if an arsenate reductase is present in GFAJ-1.

It is indeed true that arsenate reductases can distinguish between phosphate and arsenate; this has more to do directly with the difficulty of reduction of phosphate and not the "recognition" of the enzyme of arsenate. In fact, we wanted to draw the reader's attention to the idea that arsenate generally gets into a cell because of the confusion of phosphate transporters and metabolic enzymes (for review see 11) that readily use arsenate instead of phosphate. The respiratory arsenate enzymes are interesting, but beyond the scope of our paper. Furthermore, we are absolutely interested in the genetic complement of GFAJ-1 in terms of arsenate reductases (ArrA and ArsC) as well as the arsenite oxidases (Aox, Arx and AsoA). We are currently pursuing the molecular biology to determine this as well as pursing full genome sequencing for GFAJ-1.

On page 5, second paragraph, it should be mentioned that As and P was relative to dry weight. It only says: "by weight".

Corrected.

On page 7 in the middle it says: "...arsenylated proteins where AsO43- would substitute for PO43-...". Here it should probably be -AsO42- and -PO42-.

Corrected by spelling out the compounds.

There appears to be a mistake in Figure Legend 1: On line 7, panels (A) and (B) should probably be (B) and (C), respectively.

Corrected.

Acknowledging the fact that GFAJ-1 still grows better on phosphate than arsenate as mineral nutrient, I suggest the title to be refraised to:
"Arsenolife: A bacterium that CAN GROW by employing arsenic instead of phosphorous". In the same context, it would be very valuable for understanding the system, if an arsenate reductase is present in GFAJ-1 and if, how it is regulated.

We have edited the title, as per the suggestion of this reviewer and the managing editor at Science, and again reiterate that we are pursuing the molecular biology and genetics to determine if the other "normal" arsenic related genes including arsenate reductases and arsenite oxidases are present. However, we will point out that our EXAFS data did not show arsenite accumulation or other reduced arsenic species that would have resulted from the activity of the former enzyme family.

Review 2

The manuscript by Wolfe-Simon et al. is well written, concise, to the point and provides exciting and novel results. The authors provide many lines of evidence to prove their point that the isolated novel bacterium (at least to some extent) can replace phosphate by arsenic in its biomolecules. It's a pleasure to get a well-conceived and carried-out study to review. However, before publication the following points need to be considered:

1) In order to demonstrate that the isolated strain can really grow continuously replacing P by As, it has to be demonstrated that the culture can be transferred several times (5-10 times) without adding any phosphate still showing an increase in OD and cell number. I would recommend providing cell numbers or OD values of cultures after each transfer step. This leads to a question regarding Fig. 1B: in this case, was the culture inoculated

from a –As/+P culture or from a +As/-P culture? If it was a –As/+P culture, how much phosphate was added (stemming from the cells in the inoculum) when inoculating the fresh medium?

The strain GFAJ-1 was picked as a colony off a plate grown +As/-P along with more than ten other candidate isolates (all separately maintained in liquid culture). All of these isolates are maintained in the lab under +As/-P conditions and currently are more than 14 transfers from the plate picked stage (we also made this more clear in the SOM). We transfer the isolates approximately every two to four weeks, when ODs are generally between 0.4 to 0.8, in a 1:10 dilution from +As/-P to +As/-P. All of the inocula for all of the experiments shown were grown +As/-P. Thus, the cells can be grown long term, for multiple generations from the picked colony stage with no added phosphate (See ICPMS table in SOM for details on the low background P in our lab). Before this, they were grown as an enrichment for more than 10 transfers and always into new medium that was +As/-P. We therefore feel that there is no serious carry over and we work to maintain as close to a P-free environment in the lab reagents and materials as possible.

2) Can the authors rule out that the cells recycle an internal pool of phosphate after inoculation from –As/+P medium into +As/-P medium? Could degradation of ribosome provide enough P to sustain growth?

As described above, all experiments are initiated with inocula from +As/-P condition. The +As/-P is the condition the cells are maintained under at all times in the lab. Cells are only grown with added P for experiments. Thus we do not feel this is a serious issue given the reproducible growth we obtain but would be an interesting experiment to follow up with.

3) What was the lowest concentration of phosphate that sustains similar growth as observed in the +As/-P medium?

We have no evidence for the answer to this question yet. While this is indeed an interesting experiment, which we intend to pursue for our next publication, in this report we wanted to establish this physiological phenomenon and present to the community these data. Further experiments will be extremely critical to help understand the interplay between P and As both alone and in tandem in the medium.

4) For synthesis of DNA, nucleoside triphosphates react by splitting of pyrophosphate and releasing enough energy necessary for the continuation of the DNA strain. In the case of As replacing P, this would require nucleoside triarsenates and the release of an arsenate dimer. Is there any evidence for trimers or dimers of arsenate? Maybe a theoretical consideration of the energetics of such reactions would help to better evaluate whether such reactions are feasible at all. How could otherwise the synthesis of the biomolecules work energetically?

The reviewer makes an excellent point some of which we explored in an earlier conceptual paper (1). As we point out in that paper there are reports in the literature showing spontaneous formation of compounds like adenosine mono arsenate (see for example 9) as well as the kinetics of pyroarsenate hydrolysis (2). In addition to polyesters of arsenate, the volatile mono arsenate ester bond may contain enough energy to facilitate DNA synthesis and we are eager to pursue measurements of this in the future. One report suggested that protein biosynthesis could potentially occur with arsenylated esters in mitochondrial preparations (12).Others have show that enzymes can indeed form and/or recognize ADP-As (13-16) as well as other analogs such as glucose-6-arsenate (14, 15) and arsenopyruvate (17). We feel that the polyarsenate may or may not be absolutely required for this physiology to occur. However, we are excited to determine in the future the exact mechanism that this organism is using to accomplish what our data show- that is arsenate in a variety of biomolecules suggesting replacement of phosphate.

5) In order to demonstrate and quantify the replacement of P by As, I recommend isolating DNA/RNA and determining the element ratio of C, N, As to demonstrate and quantify to which extent P was replaced by As.

We have included a new figure (new Figure 2) that addresses this issue. Here we present evidence of extracted gel purified genomic DNA from +As/-P cells that contains As as compared to -As/+P cells. Although our data suggest that it still includes some P, we argue that this is in agreement with the other evidence we have, that is that the cells are scavenging some very small quantity of P. We also argue that it is not enough for cellular maintenance. Pursuing the extent of substitution and how is planned for our next set of publications. Here we strive to show that the cells appear to be utilizing the As in an assimilatory way, in particular in DNA.

6) Figure 3: the individual cells (SEM image in Fig. 1) are much smaller than the structures shown in figure 3. Are these aggregates of cells (similar to the one seen in the SEM image)? Maybe the authors can describe in the figure legend what exactly is seen in the figure.

The structures shown in Fig. 3 are indeed aggregates of cells and correspond to the resolution indicated in the scale bar for that figure as compared to Fig. 1C, D and E where the resolution is much finer. The figure legend has been expanded for clarification.

7) Figure 3: in order to localize/identify the cells, maps of C, N and S should be shown as well. Is this possible?

In addition to a new figure (Fig. 2) that addresses comment 5 above, this figure also addresses this excellent point. As our paper was in review, we pursued an extensive array of NanoSIMS analyses. Here we now show cellular ion ratio maps of $^{75}As^-:^{12}C^-$ and $^{31}P^-:^{12}C^-$. As you can see, the +As/-P cells are low with respect to P:C but contain regions of high As:C. In contrast, -As/+P cells show high and widely distributed P:C and low As:C. The latter As:C ratio is not surprising because all of our cultures are inoculated from +As/P cells (see explanation in previous responses to comments #1 and 2) and so some carryover of As is expected. Figure 3 compliments this showing biologically required metals corresponding to cellular arsenic at a lower resolution.

8) Figure 3: the correlation between Zn and Fe is pretty obvious in the figure. In contrast, the As map is not always consistent with the Fe and Zn intensities. This should be mentioned in the text or legend.

Now included in legend. Additionally, we now include in the SOM correlation plots showing the relationship between these elements from these data (Fig. S3).

9) Figure 2: the % values, do they refer to the amount of 73As present initially? The remaining As (if you sum up all % values in figure 2), is this aqueous (free) arsenate? If not, how much free arsenate (in ionic form) is present in this experiment?

The % values refer to the total amount of isotope recovered in biological samples- we measured the initial cell pellet and then the decay associated with each fraction. We had almost 98 % recovery of the total isotope associated with the whole cell biomass between all the various fractions. Cells were washed multiple times with cold arsenate to assure us that the isotope measured is indeed intracellular to the biomass and was confirmed given the distribution of the isotope in the various fractions recovered. The total free arsenate in the experimental cultures was 20 mM "cold" AsO_4^{3-} with the addition of 2 $\mu Ci\ ml^{-1}$ "hot" arsenate to each replicate.

10) Figure 3A: the EXAFS signal fits nicely to the theoretical EXAFS data calculated for As-DNA. However, as shown in Figure 2, most of the 73As is in the protein fraction. Does that mean that the As in the proteins give a similar signal as the As in the DNA? How does this look like for P in DNA and P in proteins (or in a "normal" protein fraction)?

This observation is correct! In fact, we would expect protein arsenylation to be similar to the bond lengths in nucleic acids. We have now included an expanded table (Table S3) of bond

length distances in the SOM to provide this for the reader as well as bond lengths for other important P-containing biomolecules (See below in response to Reviewer 3 comment 3). Our next set of experiments will be part of an indepth study using EXAFS among other techniques to explore this question with additional data.

Review 3

Reviewing this paper was a rare pleasure. It is clearly-written and well-reasoned. The authors chose the right methods, designed the right experiments, obtained solid data supporting the conclusion that GFAJ-1 uses As in place of P. They use appropriate caution in interpreting results. I think the paper is just about publishable as is; my comments for revision are below. Great job! I look forward to seeing follow-up work in the future.
Comments that likely require revision:
1) I would like to see more explanation (probably in the SOM) of the EXAFS results, as compared to the structure of DNA.

We used DNA initially as a biomolecule for reference, here we are really just noting that our EXAFS is consistent with the biological properties of these types of molecules. Clearly, our future work will use purified compounds from +As/-P cells and will be presented in a followup paper. We have edited Fig 3 to reflect that this is a fit of a mixture of compounds and included a new table in the SOM with bond lengths for comparison (Table S3).

-Why were only a sulfide and arsenide used as standards? Why not arsenate minerals?

These two compounds were used for comparison because they are very common forms of biologically altered arsenic. For example, arsenate reduction to detoxify the intracellular arsenic. We show that it isn't reduced. We were in particular concerned to show that the arsenate was not an artifact and simply adsorbed to the cell surface. The arsenic minerals would only indicate As-As bonds which our data do not show. Other minerals like As_2O_5 would not be as relevant in this case. This was not meant to be an exhaustive comparison but rather illustrative for comparison of bond lengths and peak intensities.

-It's nice to see the As DNA fit in Fig 3A, but it would also be useful to have a compilation of the As-X distances mentioned (and considered as possibilities), as well as the P-O and P-C distances in DNA and the other molecules mentioned (e.g. NADH, ATP, acetyl-CoA…not sure how glucose would be substituted, though). This way the reader can evaluate for him/herself whether the distances are "consistent." For instance, in my computer model of DNA, I do see 2 P-C distances ~2.3A but only 1 P-C that is close to 3A. Anyway, not everyone will have a model (or other such information) handy, so a table w/ references will help support interpretations.

This is an excellent point and so we have included this table in the SOM (Table S3). Here you can now see that our EXAFS on whole cell preparations does indeed seem to represent a mixture of As compounds that mimic known ones that typically contain P.

-Are the P-O and P-P distances of polyphosphate known? Could there be a polyarsenate? Even if so, I suspect this can be ruled out by the EXAFS data.

Inclusion of an As-As bond distance in the EXAFS fit does not significantly improve the fit quality however, this doesn't rule it out. Based on the level of data quality, it is not justified to include here. Further studies using purified compounds will be used to revisit this issue.

2) I would like to see a table with more detailed ICP-MS data in the SOM, including results from the controls.

We have included, as Table S1, these data in the SOM.

Comments that do not necessarily require revision:
3) I am curious about the wide range intracellular As concentrations in +As/-P cultures. I am also wondering why there is < one third of the As content (by weight, so even less by atomic ratio) in the +As/-P cultures as compared to the –As/+P cultures. Are you implying that the "vacuole" might represent a place of As storage (which might cause large variations)?

This is a great comment and of interest for future investigation. We are curious what is in the large "vacuole-like" structure and wonder that if As-DNA is unstable as compared to P-DNA if these regions contain some sort of non-aqueous hydrocarbon if the As-DNA would be more stable there. We look forward to finding this out! Overall, we do attribute the wide variation of cellular As to natural variation. These cells are all in stationary phase and may well display differences in elemental stoichiometry overall. That said, the physiological differences between +As/-P and -As/+P cells may be wide ranging and we look forward to teasing this problem out. This report was to demonstrate this phenomenon to the community and open up this for other groups to also begin to investigate.

Minor comments that may require revision:
4) I don't think I would write "vacuole" when it is likely (and interpreted) that a solid inclusion was dissolved in the process of TEM sample preparation. This is somewhat misleading.

We are not sure what else to call this region in the TEMs. We have edited the text to say "vacuole-like" but would like to point out that these do not seem to be an artifact of preparation

because we see evidence of these structures in the "plump" nature of the +As/-P cells in our SEM images and also by light microscopy during AODC data collection (not shown).

5) Figure 3A: From the caption text, I am not entirely sure what spectrum corresponds to what model compound.

We have indicated on the figure itself which spectrum belongs to which compound. For further clarification, we now explain this in the figure legend.

Correlation plots corresponding to 3B (e.g. As vs. P, As vs. Fe, As vs. Zn) would be helpful (in SOM) but not absolutely necessary.

We have now included these correlation plots in the SOM, see Fig. S3.

6) "X-ray spectroscopy cannot detect light elements like carbon" – this statement is not exactly true, and should be qualified appropriately given that you "see" (or may see) carbon in your results, and other X-ray spectroscopy methods can actually be specific for carbon (C-NEXAFS).

We have edited the text to clarify and address this issue. Our lightsource experiments were done under non-vacuum conditions and we used X-ray fluorescence thus we were not able in our work to "see" carbon. Nicely, our NanoSIMS data now compliments the EXAFS work and includes carbon.

References for Responses to Editor and Reviewers:

1. F. Wolfe-Simon, P. C. W. Davies, A. D. Anbar, *Int J Astrobio* **8**, 69 (2009).
2. T. G. Richmond, J. R. Johnson, J. O. Edwards, P. H. Rieger, *Aust. J. Chem.* **30**, 1187 (1977).
3. C. D. Baer, J. O. Edwards, P. H. Rieger, *Inorg. Chem.* **20**, 905 (1981).
4. J.-M. Crafts, *Bull. Soc. Chim. Fr.* **14**, 99 (1870).
5. A. M. Schoffstall, *Origins of Life and Evolution of Biospheres* **7**, 399 (1976).
6. A. M. Schoffstall, R. J. Barto, D. L. Ramos, *Origins of Life and Evolution of Biospheres* **12**, 143 (1982).
7. A. M. Schoffstall, E. M. Laing, *Origins of Life and Evolution of Biospheres* **15**, 141 (1985).
8. S. A. Benner, A. Ricardo, M. A. Carrigan, *Curr Opin Chem Biol* **8**, 672 (2004).
9. R. Lagunas, D. Pestana, J. C. Diez-Masa, *Biochemistry* **23**, 955 (1984).
10. J. Berg, J. Tymoczko, L. Stryer, *Biochemistry*. (WH Freeman & Co, New York, ed. 6th, 2007).
11. B. Rosen, *FEBS Lett* **529**, 86 (2002).
12. T. Ozawa, M. Hagihara, N. Yamanaka, K. Yagi, *Arch. Biochem. Biophys.* **137**, 585 (1970).
13. M. Avron, A. T. Jagendorf, *J Biol Chem* **234**, 967 (1959).
14. L. Slooten, A. Nuyten, *Biochim Biophys Acta* **725**, 49 (1983).
15. M. J. Gresser, *J Biol Chem* **256**, 5981 (1981).
16. S. A. Moore, D. M. Moennich, M. J. Gresser, *J Biol Chem* **258**, 6266 (1983).
17. S. Chawla, E. K. Mutenda, H. B. Dixon, S. Freeman, A. W. Smith, *Biochem. J.* **308**, 931 (1995).